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FULL PAPER

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Agrobacterium tumefaciens-mediated genetic transformation of the white root rot ascomycete *Rosellinia necatrix*

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Abstract Hygromycin B resistance was conferred to the mycelium of the white root rot fungus *Rosellinia necatrix* by transformation with the hygromycin B phosphotransferase gene (*hph*) of *Escherichia coli* under the control of the heterologous fungal *Aspergillus nidulans* P-*gpd* (glyceraldehyde 3-phosphate dehydrogenase) promoter and the *trpC* terminator. In all three transformants, the presence of *hph* and single-copy integrations of the marker gene were demonstrated by Southern analysis. This is the first report describing *A. tumefaciens*-mediated transformation of *R. necatrix*

Key words *Agrobacterium tumefaciens* · Hygromycin B · *Rosellinia necatrix* · Transformation

Introduction

Filamentous ascomycetes *Rosellinia necatrix* (Hartig) Berlese is a commercially important, soil-borne, root pathogen affecting a wide range of plant species. It is the causal agent of white root rot disease, and host plants infected by the fungus quickly wither and die. In Japan, this fungal disease, which spreads rapidly and is very difficult to prevent, has done great damage to commercially grown grapevines, apple and pear trees, and other crops. Unfortunately, despite its importance, little is known about heterokaryon formation by this fungus. Previously, we reported genetic differences among single ascospore cultures isolated from the same perithecium using some restriction fragment length polymorphism markers, which were newly amplified and cloned as a single-copy gene, and random amplified polymorphic DNA markers. Based on these results, the life cycle of *R. necatrix* appears to be heterothallic (Kanda et al. 2003). However, the vegetative incompatibility and mating system in *R. necatrix* are not well understood (Aimi et al. 2002).

The development of a suitable transformation protocol for *R. necatrix* is essential to allow genetic manipulation for disease control and for investigation of heterokaryon formation. Here, we describe procedures for the genetic transformation of mycelium from *R. necatrix* by applying an *Agrobacterium tumefaciens* (Smith and Townsend) Conn-mediated transformation system. An *A. tumefaciens*-mediated transformation system in fungi has been reported and is a powerful tool for the genetic manipulation of filamentous fungi (Piers et al. 1996; de Groot et al. 1998; Gouka et al. 1999; Malonek and Meinhardt 2001; Mikosch et al. 2001; Rho et al. 2001; Zwiers and De Waard 2001). This is the first report of the genetic transformation of *R. necatrix*, a system that provides a powerful means for genetically manipulating this phytopathogenic fungus.

Materials and methods

Strains and culture conditions

The field-isolated strains (W4, W20, W30) of *R. necatrix* used in this study (Table 1) were originally obtained from Dr. Naoyuki Matsumoto (National Institute of Agro-Environmental Science, Japan) and were maintained on 1/5 oatmeal agar (OMA) medium (12 g/l oatmeal and 20 g/l agar). Mycelium for DNA extraction was grown on a cellophane sheet overlaid on potato dextrose agar (PDA) (potato extract, 2% glucose, 2% agar). The *A. tumefaciens* strain LBA4404 was routinely grown on Luria-Bertani (LB) agar (1% sodium chloride, 1% tryptone, 0.5% yeast extract, pH 7.0) containing 50 μg/ml kanamycin to maintain the plasmid pAN26-CAMBIA1300 for transformation experiments.

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Table 1. *Rosellinia necatrix* strains used in this study

Strain	MCG[a]	Characteristics	Location	Source
W4	3	Field-isolated original strain	Okayama, Japan	Dr. Matsumoto
W20	12	Field-isolated original strain	Okayama, Japan	Dr. Matsumoto
W30	19	Field-isolated original strain	Chiba, Japan	Dr. Matsumoto

[a] MCG, mycelium compatibility group

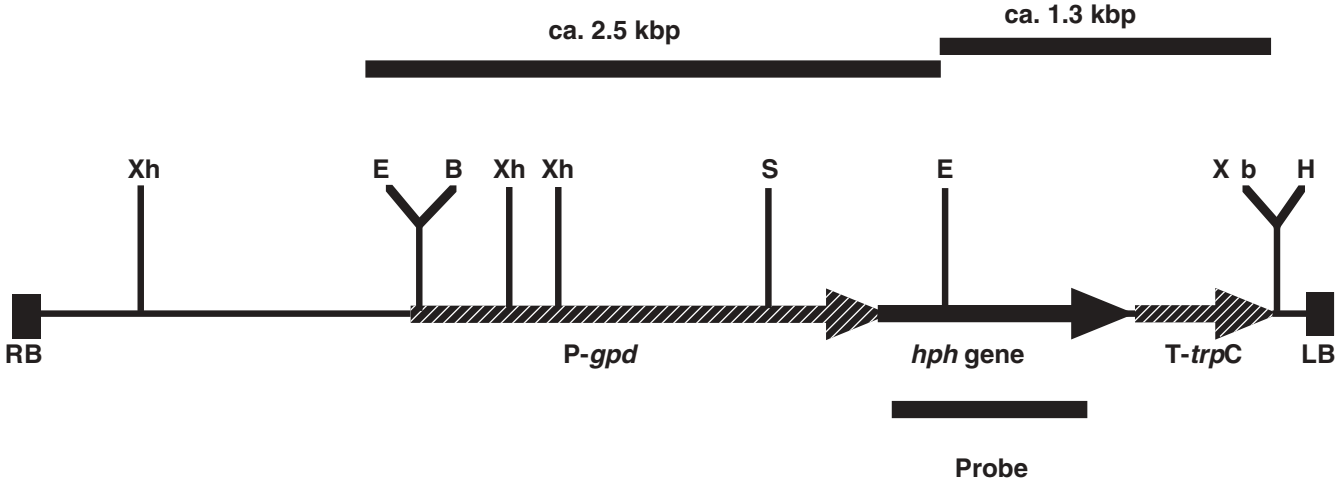

Fig. 1. Physical map of the hygromycin B-resistant cassette of pAN26-CAMBIA1300. A selection of restriction sites is shown: (*B*, *Bam*HI; *E*, *Eco*RI; *H*, *Hin*dIII; *S*, *Sal*I; *Xb*, *Xba*I; *Xh*, *Xho*I). *RB* and *LB* represent the right and left borders of T-DNA, respectively. *Bars* correspond with hybridization signals, which are shown in Fig. 3

Plasmid construction

The plasmid vectors pAN26 and pCAMBIA1300 were supplied by the Fungal Genetics Stock Center (Kansas City, KS, USA) and the Center for Application of Molecular Biology to International Agriculture (Canberra, Australia), respectively. The plasmid pAN26-CAMBIA1300 contains both the hygromycin B resistance cassette, which is based on pAN26 (Taylor and Borgmann 1996) for the selection of resistant fungal clones (see also Fig. 1), and a kanamycin resistance gene for selection in *Agrobacterium*. The hygromycin B resistance cassette contains the hygromycin phosphotransferase gene (*hph*) under the control of the glyceraldehyde 3-phosphate dehydrogenase promoter (P-*gpd*) and the *trpC* terminator (T-*trpC*) from *Aspergillus nidulans* (Eidam) Winter. The pAN26-CAMBIA1300 vector was constructed as follows. To remove *hph* in pCAMBIA1300, the plasmid was digested with *Xho*I and then self-ligated. The resultant plasmid was digested with *Bam*HI-*Xba*I and ligated with the *Bam*HI-*Xba*I fragment of pAN26 containing the hygromycin B resistance cassette. Using a triparental mating procedure with the helper plasmid pRK2013 (Ditta et al. 1980), the pAN26-CAMBIA1300 construct was transferred from *Escherichia coli* (Migula) Castellani and Chalmers to *A. tumefaciens* LBA4404 (Bevan 1984).

A. tumefaciens-mediated fungal transformation

The transformation procedure is based on the protocol described by Chen et al. (2000), with some modifications. Briefly, the *A. tumefaciens* strain LBA4404 carrying the plasmid pAN26-CAMBIA1300 was grown in 5 ml minimal medium (MM; 2.0 g/l glucose, 2.05 g/l K_2HPO_4, 1.45 g/l KH_2PO_4, 0.15 g/l NaCl, 0.50 g/l $MgSO_4 \cdot 7H_2O$, 0.10 g/l $CaCl_2 \cdot 6H_2O$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$, and 0.5 g/l $(NH_4)_2SO_4$) (Hoykaas et al. 1979) containing 50 μg/ml kanamycin for 2 days at 28°C. One milliliter of the fresh culture was transferred to 100 ml MM with kanamycin and grown overnight at 28°C to an optical density at 600 nm of 0.5–0.8. Bacteria were collected by centrifugation and resuspended in induction medium [IM; 10 mM glucose, 2.05 g/l K_2HPO_4, 1.45 g/l KH_2PO_4, 0.15 g/l NaCl, 0.50 g/l $MgSO_4 \cdot 7H_2O$, 0.10 g/l $CaCl_2 \cdot 6H_2O$, 0.0025 g/l $FeSO_4 \cdot 7H_2O$, 0.5 g/l $(NH_4)_2SO_4$, 0.5% glycerol, 200 μM acetosyringone, and 40 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 5.3] (Bundock et al. 1995) to an optical density at 600 nm of 0.5. The bacterial suspension was incubated for 3–6 h at room temperature with shaking at 120 rpm to preinduce the virulence of *A. tumefaciens*.

The *R. necatrix* strains were grown in PD (potato extract and 2% glucose) medium at 25°C for 1 week. The culture broth containing the mycelial mat was homogenized with a

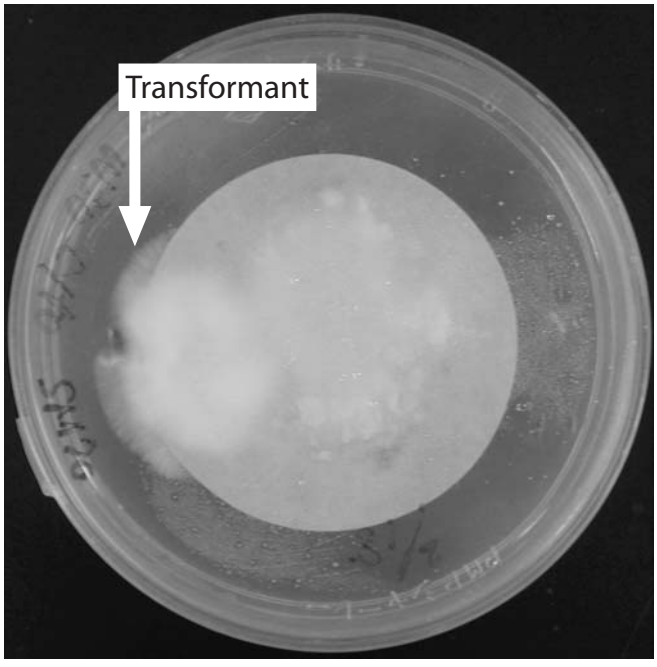

Fig. 2. Selection of putative hygromycin-resistant transformants of the *Rosellinia necatrix* W20 strain. Homogenized mycelium was cocultivated with the *Agrobacterium tumefaciens* strain LBA4404 carrying the vector pAN26-CAMBIA1300, which contains the hygromycin phosphotransferase (*hph*) construct under the control of the *Aspergillus nidulans* P-*gpd* promoter and the *trpC* terminator. Shown is the appearance of the cultures after 2 weeks on selection medium (SM) containing 80 μg/ml hygromycin B

Nissei Homogenizer (AM-12) at 8000 rpm for 2 min. The culture broths containing the homogenized mycelium and the virulence-preinduced *A. tumefaciens* were mixed and collected by centrifugation**.** The resultant pellet containing *R. necatrix* mycelium and *A. tumefaciens* was transferred onto filter paper, overlaid on IM agar medium, and then cocultivated at 25°C for 3 days. Next, the filter paper was transferred onto selection medium [SM; potato dextrose agar (PDA) containing 80 μg/ml hygromycin B and 200 μM cefotaxime]. Hygromycin-resistant colonies appeared at the margins of the filter paper after approximately 2 weeks (Fig. 2), after which the mycelium were transferred to and maintained on PDA containing 80 μg/ml hygromycin B.

DNA extraction

DNA was extracted from lyophilized tissue of the *R. necatrix* strains harvested from PDA using the CTAB (hexadecyltrimethylammonium bromide) procedure essentially as described previously by Zolan and Pukkila (1986). The extracted DNAs were suspended in 20 μl TE [50 mM Tris-HCl, 10 mM ethylenediaminetetracitic acid (EDTA), pH 8.0] and then separated by electrophoresis in 1% agarose gels with 0.1 μg control lambda DNA. The gels were stained with 0.5 μg/ml ethidium bromide and photographed under UV light. The amount of each DNA present was estimated by comparing the ethidium bromide fluorescence intensity of the control lambda DNA with that of the *R. necatrix* DNA.

Southern hybridization

Genomic DNA from *R. necatrix* strains was isolated and digested with the appropriate restriction endonucleases, separated by agarose gel electrophoresis, and blotted onto Hybond-N+ nylon membranes (Amersham Biosciences, Piscataway, NJ, USA). The membranes were probed with DNA fragments using the Gene Image random primed nucleic acid labeling and detection system (Amersham Biosciences). Procedures for probe labeling, hybridization, and detection were carried out according to the manufacturer's recommendations.

To amplify the DNA of *hph* used as the hybridization probe, the oligonucleotide primers hphF (5′-GATATGA AAAAGCCTGAACTC-3′) and hphR (5′-TTCCTTTG CCCTCGGACGAGT-3′) were designed based on the nucleotide sequence of *hph* in pAN26. Polymerase chain reaction (PCR) was carried out in a 100-μl reaction mixture containing 1 × ExTaq buffer, 1 ng plasmid DNA (pAN26), 100 pmol of each primer, deoxynucleoside triphosphale (dNTP) at 0.2 mM each, and 2.5 U ExTaq polymerase. The reaction was performed for 30 cycles using the following cycling profile. The first denaturing step was at 94°C for 2 min, followed by PCR cycles of 30 s of denaturation at 94°C and 2 min of annealing and extension at 68°C. The amplified DNA fragments encoding *hph* were purified with a Microcon-100 filter (Millipore, Bedford, MA, USA) and used as a DNA template for direct sequencing and as a probe for Southern hybridization. DNA sequencing was carried out in an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Branchburg, NJ, USA) by chain-termination procedure with a BigDye Terminator cycle sequencing kit (Applied Biosystems) using the hphF and hphR primers.

Results and discussion

Minimum inhibitory concentration of hygromycin B in *R. necatrix* strains

The growth inhibition by hygromycin B of wild-type strains and putative transformants of *R. necatrix* were tested by plating a mycelial agar block on complete PDA medium supplemented with various concentrations of hygromycin B (0, 60, 70, 80, 100, 120, 150, 180, and 200 μg/ml). The growth of the wild-type strains was completely inhibited on medium containing 80 μg/ml hygromycin B. Therefore, we used this concentration for the selection of resistant colonies (Table 2).

Transformation experiments and characteristics of putative transformants

In five independent experiments, we observed resistant fungal clones of the W20 and W30 strains on SM. However, we

Table 2. Growth inhibition of *R. necatrix* by hygromycin B

Strain	Hygromycin B (μg/ml)								
	0	60	70	80	100	120	150	180	200
Wild-type									
W4	+	+	+	−	−	−	−	−	−
W20	+	+	+	−	−	−	−	−	−
W30	+	+	−	−	−	−	−	−	−
Transformant									
W20-26-T1	+	+	+	+	+	+	+	+	−
W20-26-T2	+	+	+	+	+	+	+	−	−
W30-26-T1	+	+	+	+	+	+	+	+	+

+, could grow; −, could not grow
The mycelia were grown on potato dextrose agar (PDA) containing various concentration of hygromycin B for 3 weeks

did not obtain resistant colonies from the W4 strain (Table 2). These results might be caused by host specificity of *A. tumefaciens* and variation of sensitivity of *R. necatrix* to *A. tumefaciens*. Because we used mycelium as the host of transformation, the efficiency could not compare with that obtained with conidia as the host (Malonek and Meinhardt 2001). However, the putative transformants were obtained only from one to two of five independent experiments, and the efficiency of transformation with *A. tumefaciens* LBA4404 was low. Therefore, more transformation experiments using other *A. tumefaciens* strains are needed to increase the efficiency of transformation.

The minimum inhibitory concentrations of hygromycin B in the three transformants are shown in Table 2. All transformants could grow on PDA supplemented with 80 μg/ml hygromycin B. Moreover, the W30-26-T1 was resistant to more than 200 μg/ml hygromycin B. The growth rate on PDA supplemented with 150 μg/ml hygromycin B was 17.5 mm/week for W20-26-T1 and W20-26-T2 and 35 mm/week for W30-26-T1. On the other hand, the wild-type strains W20 and W30 could not grow on the same medium. These results suggest that the introduced *hph* under the control of the P-*gpd* promoter from *A. nidulans* is functional in the *R. necatrix* cells.

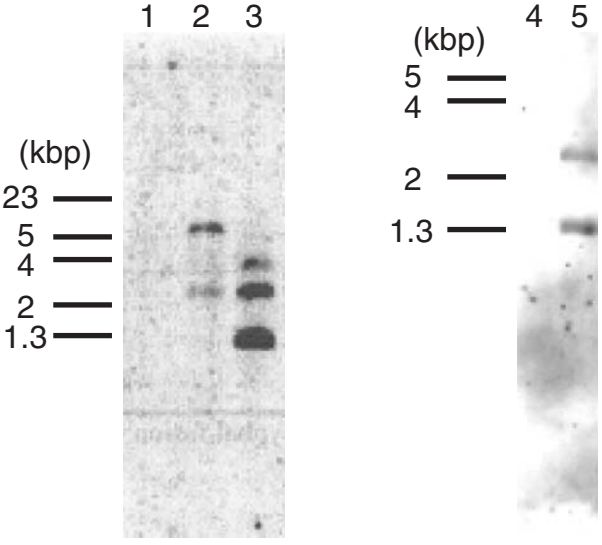

Fig. 3. Southern blot analysis of genomic DNA from *A. tumefaciens*-mediated transformants using pAN26-CAMBIA1300 as the vector. Genomic DNAs were double digested with *Eco*RI and *Hin*dIII and separated on 1% agarose gels. Hybridization was performed using amplified *hph* from pAN26 as a probe. The position and size in kilobase pairs (*kbp*) are indicated on the *left* using lambda DNA that was double digested with *Eco*RI and *Hin*dIII. *Lane 1*, wild-type strain W20; *lane 2*, W20-26-T1; *lane 3*, W20-26-T2; *lane 4*, wild-type strain W30; *lane 5*, W30-26-T1

Integration of *hph* in to the genome of *R. necatrix*

To determine the fate of the resistance gene after transformation, we performed restriction analysis of genomic DNA isolated from the three transformants followed by Southern analysis with an amplified DNA fragment of *hph* labeled with fluorescein as the probe. Genomic DNAs from wild-type strains W20 (Fig. 3, lane 1) and W30 (Fig. 3, lane 4) did not produce a hybridization signal. The *hph* probe (see also Fig. 1) has one site for the applied restriction enzyme *Eco*RI. Hence, in Southern blots, two hybridizing bands (~2.4 kbp and ~1.3 kbp; Fig. 1) were predicted for genomic DNAs from W20-26-T2 (Fig. 3, lane 3) and W30-26-T1 (Fig. 3, lane 5). These results indicate that the hygromycin B resistant cassette was integrated into the genome of W20-26-T2 and W30-26-T1 and that *hph* introduced into *R. necatrix* was expressed under the control of the P-*gpd* promoter from *A. nidulans*. In genomic DNAs from W20-26-T1 (Fig. 3, lane 2), a hybridization signal of ~2.4 kbp was predicted; however, the hybridization signal of ~1.3 kbp disappeared and a band of ~6 kbp appeared. This result suggests that the hygromycin B-resistant cassette was integrated between the *Eco*RI site in the upstream region of P-*gpd* and the *Hin*dIII site in the downstream region of T-*trpC*. Thus, the *Hin*dIII site may be removed, changing the hybridization from ~1.3 kb to ~6 kb.

To examine for the copy number of the hygromycin B-resistant cassette integrated in to *R. necatrix* genome, genomic DNAs of the transformants were digested with restriction enzymes that can cut at a single site between left and right borders of T-DNA, such as *Sal*I, and were sub-

jected to Southern hybridization using *hph* as a probe. A single hybridization signal appeared in each digest, suggesting that transformation with *A. tumefaciens* results in only single-copy integrations in each transformant (data not shown).

This is the first report of a successful transformation of *R. necatrix*. The transformation procedure was simple and gave reproducible results. The genetic manipulation of fungal genes involved in phytopathogenicity is now possible because *A. tumefaciens* transformation resulted in single-copy integrations. Moreover, using multiple rounds of integrative transformation, it should be possible to create a complete collection of insertional mutants that can subsequently be assessed phenotypically and genotypically. Thus, our findings are of great significance not only for studying the phytopathogenicity of the fungus but also for general investigations of its genetics.

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